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Buehler, D. M.; Baker, A. J.

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POPULATION DIVERGENCE TIMES AND HISTORICAL DEMOGRAPHY IN RED KNOTS AND DUNLINS

DEBORAH M. BUEHLER[1] AND ALLAN J. BAKER[2]

Center for Biodiversity and Conservation Biology, Royal Ontario Museum, Toronto, Ontario, M5S 2C6, and Department of Zoology, University of Toronto, Toronto, Ontario, M5S 3G5, Canada

Abstract. We employed Bayesian coalescent modeling of samples of mitochondrial control region sequences in two species of shorebird, Red Knots (*Calidris canutus*) and Dunlins (*Calidris alpina*) to estimate evolutionary effective population size, population divergence times, and time to most recent common ancestor of genes in the samples. The gene trees for the two species contrast sharply: knot haplotypes were connected in a shallow, star phylogeny whereas Dunlin haplotypes were related in a deeper bifurcating genealogy. Divergence times of populations representing all six subspecies of knots are estimated to have occurred within the last 20 000 (95% CI: 5600–58 000) years, and evolutionary effective population sizes of females are small (N_{ef} = 2000–14 000). We hypothesized that breeding knots were restricted to unglaciated regions of Eurasia during the last glacial maximum, and gradually expanded eastwards into Alaska, the high Canadian Arctic and Greenland as the ice melted. Population divergence times in Dunlins are much older (58 000–194 000 ybp) and effective population size has historically been higher in major lineages (N_{ef} = 12 000–44 000). We conclude that Dunlin populations were not severely reduced in size in the last 200 000 years, and major lineages have differentiated under restricted gene flow for a much longer time than knots. Knots present a snapshot of genetic evolution in the last 20 000 years, whereas Dunlins display patterns of genetic evolution over an order of magnitude longer time frame.

Key words: Calidris alpina, Calidris canutus, *coalescent theory, control region, mitochondrial DNA, phylogeography.*

Tiempos de Divergencia Poblacional e Historia Demográfica en *Calidris canutus* y *C. alpina*

Resumen. Aplicamos modelos Bayesianos de coalescencia en una muestra de secuencias de la región de control mitocondrial de dos especies de playeros, *Calidris canutus* y *C. alpina*, para estimar el tamaño efectivo de la población, los tiempos de divergencia entre poblaciones y la distancia cronológica al antepasado común más reciente de los genes muestreados. Los árboles genealógicos de las dos especies contrastan fuertemente: los haplotipos de *C. canutus* están conectados superficialmente siguiendo un patrón filogenético en forma de estrella, mientras que los haplotipos de *C. alpina* se relacionan de manera más profunda, mostrando patrones de genealogía bifurcados. Se estima que la divergencia poblacional de las seis subespecies de *C. canutus* tuvo lugar durante los últimos 20 000 años aproximadamente, y los tamaños efectivos de la población de hembras son pequeños (N_{ef} = 2000–14 000). Presumimos que la reproducción de *C. canutus* estuvo restringida sólo a regiones de Eurasia que estuvieron libres de hielo durante el último máximo glacial y se expandieron gradualmente hacia el este de Alaska, el Ártico canadiense y Groenlandia cuando el hielo se derritió. Los tiempos de divergencia poblacional en *C. alpina* son más antiguos (58 000–194 000), y el tamaño efectivo de la población ha sido históricamente más alto en los linajes principales (N_{ef} = 12 000–44 000). Concluimos que las poblaciones de *C. alpina* no mostraron reducciones serias en los últimos 200 000 años, y que sus linajes se han diferenciado por un período de tiempo mucho más prolongado que los de *C. canutus*. Los patrones encontrados para *C. canutus* representan una imagen de evolución genética ocurrida durante los últimos 20 000 años, mientras que los patrones de *C. alpina* indican la ocurrencia de evolución genética durante un período de tiempo diez veces más largo.

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[1] Present address: Animal Ecology Group, Centre for Ecological and Evolutionary, Studies, University of Groningen, P.O. Box 14, 9750 AA Haren, Netherlands.

[2] E-mail: abaker@rom.on.ca

INTRODUCTION

In recent years molecular analysis using gene trees has allowed researchers to make inferences about the demographic histories of various extant species (Baker 2002, Fedorov and Stenseth 2002, Griswold and Baker 2002) and to examine how the effects of Quaternary glaciations have left a genetic legacy in these histories (Hewitt 2000, Luttikhuizen et al. 2003). In this paper, we use Bayesian coalescent modeling to examine population divergence times and to make inferences about phylogeography in two species of shorebird, the Red Knot (*Calidris canutus*) and the Dunlin (*Calidris alpina*). Red Knots and Dunlins, a pair of closely related species, are excellent for a comparison of demographic history. They are both long-distance migrants with similar life-history traits (Piersma and Baker 2000), yet all previous molecular investigations have shown radically different phylogeographic patterns.

Red Knots are currently classified into six subspecies, each with distinct morphological traits, migration routes, and annual cycles. Available evidence from long term banding programs indicates that distinct flyways exist (Piersma and Davidson 1992) and six separate breeding areas are known to host different populations, all of which are now formally recognized as subspecies based on body size and plumage characteristics (Tomkovich 1992, Piersma and Baker 2000, Tomkovich 2001). *C. c. roselaari*, the least studied subspecies, is thought to breed in northwest Alaska and Wrangel Island, Russia and winters in the southeast United States; *C. c. rufa* breeds in the central Canadian Arctic and winters in southern Patagonia and Tierra del Fuego, Argentina; *C. c. rogersi* breeds on the Chukotsky Peninsula in eastern Russia and winters in southeast Australia and New Zealand; *C. c. piersmai* breeds on the New Siberian Islands in north central Russia and winters in northwest Australia; *C. c. islandica* breeds in northern Greenland and northeast Canada and winters in northwest Europe; finally, *C. c. canutus* breeds on the Taymyr Peninsula in western Siberia and winters in west and southwest Africa. Earlier work on Red Knots failed to distinguish geographically isolated groups indicating an apparent panmixia caused by a late Pleistocene bottleneck (Baker et al. 1994, Piersma 1994), but analysis has been complicated by an extreme lack of genetic variability making it difficult to distinguish between ancestral polymorphism from a recent bottleneck and current gene flow. In this paper, we use additional mitochondrial DNA (mtDNA) sequence data and new statistical techniques that address these issues.

Dunlins comprise five morphologically and genetically distinct lineages (based on mtDNA) found in Canada, Europe, central Siberia, Beringia, and Alaska (Wenink et al. 1993, Wenink et al. 1996). The Canadian lineage comprises *C. a. hudsonia*, the European lineage contains *C. a. arctica*, *C. a. schinzii*, and *C. a. alpina*, the central Siberian lineages comprises *C. a. centralis*, the Beringian lineage represents *C. a. sakhalina*, and finally the Alaskan lineage encompasses *C. a. articola* and *C. a. pacifica*. For comparative purposes, we will consider only lineages based on mtDNA in Dunlins rather than population samples. Maps showing the breeding areas of Red Knots and Dunlins are shown in Figure 1a, b, respectively.

The study of evolution at the tips of evolutionary trees brings new challenges. When studying intraspecific phylogenies rather than species phylogenies, there is a higher probability that haplotypes drawn from the populations will not have had time to sort into distinct lineages (Avise 1994). The result is polyphyly or paraphyly within species. Traditional models of population subdivision assume that populations have reached equilibrium between migration and drift; however, a species undergoing lineage sorting is not in equilibrium and thus models that do not assume equilibrium are necessary. Coalescent theory has experienced rapid development over the last few years (Kingman 1982, Hudson 1990, Griffiths and Tavaré 1997, Fu and Li 1999), and out of this period of development many analytical methods have emerged. Nielsen and Wakeley (2001) describe a coalescent model that jointly estimates divergence time and migration rates for populations not necessarily in genetic equilibrium using DNA sequence data. We used the Nielsen and Wakeley (2001) model in this study to analyze mitochondrial DNA sequences from the control regions of Red Knots and Dunlins. The sequences were first used to make inferences about the historical demography of the two species. We then examined the relative importance of nonequilibrium conditions (or shared ancestral polymorphisms) among populations versus gene flow on the

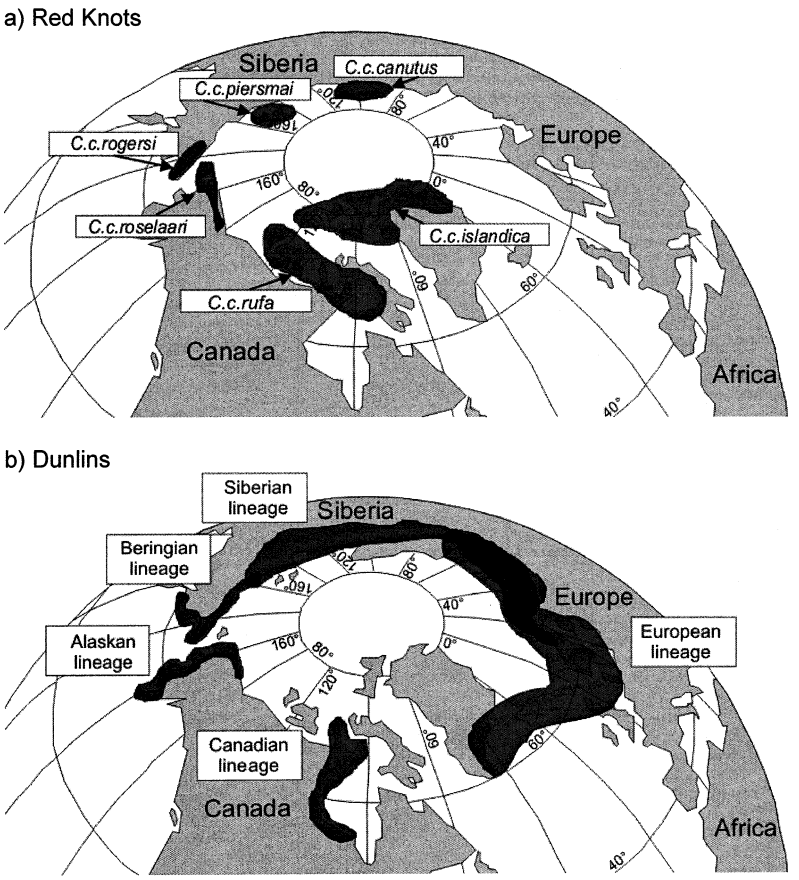

FIGURE 1. Arctic breeding grounds for a) Red Knot subspecies (Piersma and Davidson 1992) and b) Dunlin mtDNA lineages (Wennerberg et al. 1999). Land masses are shaded light grey and breeding areas are shaded in translucent dark grey. In the Dunlin distribution recent overlaps in breeding distributions are indicated where the translucent grey shading overlaps.

polyphyly or paraphyly of lineages in the species.

METHODS

SAMPLE COLLECTION

Red Knots were caught using ground traps in breeding areas, and cannon nets and mist nets in nonbreeding areas globally. Approximately 100 μL of blood were taken from each bird and stored at room temperature in 50 mM EDTA and 70% or 95% ethanol, then subsequently frozen at −80°C in the Ornithology collection of the Royal Ontario Museum.

Where possible, samples were taken from breeding birds (for half of the *C. c. rufa* and *C. c. islandica* samples); however, as knots breed in extremely low density and their nests are notoriously difficult to find, where samples from breeding birds were hard to obtain, birds were sampled in staging or wintering areas (Appendix A). Different subspecies of Red Knots breed in separate areas of the arctic tundra, and for the most part use different migratory flyways with very little overlap between subspecies. During the course of their migrations some subspecies share certain staging areas; for example, *C. c. rufa* and *C. c. roselaari* share the southeastern U.S.A. and Delaware Bay during northward migration, *C. c. islandica* and *C. c. canutus* both use the Wadden Sea in Europe, and *C. c. piersmai* and *C. c. rogersi* occur in northwest Australia during southward migration, *en route* to separate wintering areas, but the timing of their passage is quite distinct. Wintering areas are widespread and banding studies have shown that subspecies are not mixing on the wintering

grounds (Piersma and Davidson 1992). The subspecific assignment of birds not caught on the breeding grounds or on well-established wintering areas was determined using the timing of passage and their primary molt status. For example, knots found in staging areas on the Wadden Sea in mid-October are most certainly *C. c. islandica* as most *C. c. canutus* have left the Wadden Sea by mid-September (Nebel et al. 2000, Boyd and Piersma 2001b). Knots found in South Carolina and Georgia in mid-April are *C. c. roselaari* as *C. c. rufa* arrive later in the spring. During fall passage, *C. c. roselaari* molt their primary feathers, whereas *C. c. rufa* birds do not normally molt as they still need to cross the Caribbean Sea to South America (B. A. Harrington, pers. comm.).

DNA SEQUENCING

Total DNA from each bird was isolated using standard phenol/chloroform extractions (Sambrook et al. 1989). For knots, 91 individuals representing all six morphologically recognized subspecies were sequenced. Product from the 5′ end of the control region was obtained by standard polymerase chain reaction (PCR) amplification (Mullis and Faloona 1987) using primers L98 and H401 (Wenink et al. 1993) and was sequenced with H401. Product from the 3′ end of the control region was obtained using primers L438 (Wenink et al. 1993) and H1537 (5′-TGA CCG CGG TGG CTG GCA CAA G-3′, O. Haddrath, pers. comm.) and was sequenced with a newly designed primer KnotMidCRL (5′- GCA ACG GGT GAA TAC AAT CTA AGA C-3′). The amplification protocol was a 2 min denaturation at 94°C, followed by 36 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 90 sec, with a final extension at 72°C for 5 min (PerkinElmer 480 Thermocycler, PerkinElmer Life and Analytical Sciences Inc., Boston, Massachusetts). Sequences were obtained using the Sanger dideoxy chain termination method (Sanger et al. 1977) and the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation, Cleveland, Ohio) with P^{33} on 6% polyacrylamide gels. To confirm that the sequences were mitochondrial and not nuclear in origin, all sequences were aligned to the complete control region sequence presented in Buehler and Baker (2003), and were obtained using the same primers. The 208 sequences from Dunlins worldwide were obtained from datasets published in Wenink et al. (1996) and Wenink and Baker (1996), but which have not been previously analyzed together in a coalescent framework.

STATISTICAL ANALYSES

Preliminary sequence analyses were performed using MODELTEST 3.06 (Posada and Crandall 1998) to determine which model of sequence evolution best fitted the data. Variation in knot control region sequences was modeled best by either the HKY (Hasegawa et al. 1985) or the TrN (Tamura and Nei 1993) model. Dunlin sequences best fitted a variation of the TrN model that allowed for two different rates for transitions and two separate rates for transversions. To correct for rate variation among sites, the model for Dunlins had a gamma shape parameter of $\alpha = 0.61$. Minimum spanning networks were created using the TrN model in the program ARLEQUIN (Schneider et al. 2000). ARLEQUIN was also used to calculate Tajima's (1989) test of departure from neutrality, and nucleotide diversity $\pi = \Sigma \pi_{ij}(n[n - 1]2^{-1})^{-1}$ where π_{ij} equals the proportion of the nucleotide differences between the *i*th and the *j*th sequences and *n* is the number of individuals (Nei 1987). Pairwise mismatch distributions which examine the frequency of base pair changes between pairs of haplotypes were computed to check for demographic contractions and population growth using the program DNAsp3.5 (Rozas and Rozas 1999). The mismatch distribution is usually multimodal in samples drawn from populations that are in demographic equilibrium, but is unimodal for haplotypes drawn from a population that has undergone a recent demographic expansion (Slatkin and Hudson 1991, Rogers and Harpending 1992). The sample of *C. c. canutus*, which is the most variable and divergent of all subspecies, and the only sample that converged on parameter estimates, was also tested for sudden population expansion using the mismatch-distribution method (Schneider and Excoffier 1999) in ARLEQUIN. The method assumes an instantaneous stepwise expansion model from a population of N_0 to N_1 individuals *t* generations ago, followed by rapid attainment of demographic equilibrium. Three parameters were estimated: $\mu_0 = 2\mu N_0$, $\mu_1 = 2\mu N_1$, and $\tau = 2\mu t$, where μ is the mutation rate for the locus. Parametric bootstrapping was used to test the fit of the model based on the sum of square deviance (SSD)

between the observed and expected mismatch distributions. If acceptable model fit is confirmed, the time since the putative expansion event can be estimated from $\tau = 2\mu t$.

Coalescent analyses of Red Knot subspecies and Dunlin mtDNA lineages were performed using the model of sequence evolution (Hasegawa et al. 1985) in the program MDIV (Nielsen and Wakeley 2001). MDIV is based on a coalescent model that jointly estimates the divergence time and migration rates among pairs of populations using DNA sequence data. This method is considered superior to conventional F_{ST}-based methods because it simultaneously estimates, from the same dataset, several parameters of interest: θ, *M*, *T*, and *TMRCA*. The parameters are measures of mutation $\theta = 2 N_{ef} \mu$, migration rate $M = N_{ef} m$ and divergence time T (or *TMRCA*) = $t N_{ef}^{-1}$ respectively, where N_{ef} is the effective population size of females, t is generation time and μ is the per locus mutation rate. The program uses Markov Chain Monte Carlo simulations to generate posterior probability distributions whose modes represent the parameter estimates. Credibility intervals for the estimates are taken as the interval that contains 95% of the posterior probability distribution (Nielsen and Wakeley 2001).

The mutation rate (μ) for the domain I portion of the Red Knot and Dunlin sequences was based on the per site mutation rate per generation for the 5′ end of the control region of the Chaffinch (Marshall 1997), as this is the same rate as that calculated for shorebirds (Scolopacidae) by Paton (2003). Domain I (5′ end) mutation rates were then adjusted to reflect lower rates of substitution in domain III (3′ end) by multiplying by the ratio of the number of variable sites in the two regions. Because MDIV requires a per locus estimate of μ the mutation rates per site for domains I and III were multiplied by their respective sequence lengths and by generation time (g) to obtain $\mu = 9.39 \times 10^{-5}$ substitutions per locus per generation. Generation time in both Knots and Dunlins was assumed to be two years (Boyd and Piersma 2001a).

Throughout the results, nucleotide diversities are presented as means $\pm$ SD and parameters estimated using MDIV are presented with 95% credibility intervals. In multi-comparison tests, significant population differentiation was assumed when $P < 0.01$.

RESULTS

NEUTRALITY TESTS

In the Red Knot control region sequences, Tajima's test showed a significant deviation from neutral expectations in the sample from *C. c. rogersi* ($D = -1.72$, $P < 0.05$) and when all subspecies were pooled ($D = -1.98$, $P < 0.01$). These negative *D* statistics indicate that there are a greater number of rare alleles present than would be expected if the species were at equilibrium. This is characteristic of species that have undergone recent bottlenecks and subsequent population expansion. Results presented in this paper and earlier research (Baker et al. 1994) indicate that Red Knots were severely bottlenecked in the late Pleistocene. In a population expanding rapidly after a bottleneck, an excess of rare alleles is expected as population growth preserves rare alleles from elimination by genetic drift. Thus, we take this negative *D* as the signature of a bottlenecked population and not as a violation of neutrality. In Dunlins, none of the Tajima's tests were significant, and thus the sequences appear to be selectively neutral.

SEQUENCE VARIATION

In Red Knots we obtained control region sequences of 675 bp for 91 individuals containing 21 variable sites with 20 transitions and a single transversion. Twenty-five haplotypes were identified worldwide in the six named subspecies (Appendix B, GenBank accession numbers: AY198147–AY198171). In Dunlins, control region sequences of 608 bp were obtained for 208 individuals and contained 52 variable sites composed of 42 transitions, 8 transversions and two indels. The sequences yielded 53 haplotypes summarized in Appendix C, GenBank accession number: L20137. Overall nucleotide diversity among all knot haplotypes was 0.002 ± 0.001 and average nucleotide diversity per subspecies was 0.002. These within-subspecies nucleotide diversities in Red Knots were on average two times lower than those of Dunlins, even though the latter are subdivided into distinctive phylogroups (Table 1).

When pair-wise differences among sequences are presented as mismatch distributions (Fig. 2a, b), the pattern in Red Knots clearly resembles the unimodal pattern expected from a population experiencing growth (Avise 2000). This graph shows that most of the sequences differ by a

TABLE 1. Nucleotide diversities (± SD) for Red Knots (*Calidris canutus*) and Dunlins (*Calidris alpina*).

Population	*n*	Nucleotide diversity
Red Knots		
C.c. canutus	12	0.0023 ± 0.0017
C.c. islandica	15	0.0016 ± 0.0012
C.c. piersmai	15	0.0020 ± 0.0014
C.c. rogersi	19	0.0012 ± 0.0010
C.c. roselaari	15	0.0021 ± 0.0015
C.c. rufa	15	0.0011 ± 0.0010
Pooled	91	0.0020 ± 0.0014
Average per population		0.0020
Dunlins		
Alaskan lineage	33	0.0024 ± 0.0016
Beringian lineage	7	0.0046 ± 0.0031
Canadian lineage	16	0.0027 ± 0.0019
European lineage	110	0.0030 ± 0.002
Siberian lineage	42	0.0053 ± 0.0031
Pooled	208	0.0160 ± 0.008
Average per population		0.0036

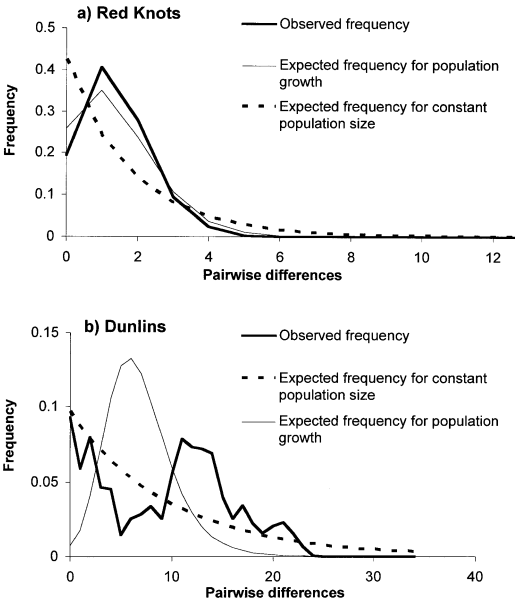

FIGURE 2. Observed and expected mismatch distributions in mitochondrial control region sequences for a) Red Knots and b) Dunlins. Red Knots closely match the pattern expected under growth conditions. Dunlins show multiple modes indicative of within and between population peaks.

single base pair change. Additionally, a mismatch analysis conducted in ARLEQUIN on the *C. c. canutus* sequences fitted a stepwise demographic-expansion model well (Schneider and Excoffier 1999), consistent with the expected unimodal mismatch distribution. We used the estimate of $\tau = 1.745$ from this analysis to date this expansion at around 18 600 (95% CI: 0, 31 270) years ago.

In contrast to Red Knots, Dunlins fit neither expectations of population growth nor constant population size, but instead have a multimodal pattern characteristic of a species with several well-differentiated groups. The two major modes in the Dunlin distribution represent within and among group differences (Fig. 2b).

GENETIC STRUCTURE

Red Knot and Dunlin haplotypes are summarized in minimum spanning networks (Fig. 3 and 4). Most haplotypes in the knot network differ by a single base change producing a star-like pattern characteristic of a species that has undergone a recent bottleneck with subsequent expansion (Slatkin and Hudson 1991). Furthermore, the most common haplotypes in the network, Rog1, Isl1, and Can1 are shared among subspecies, with the only evidence of population genetic structure coming from the clustering of *C. c. canutus* haplotypes near the top of the network (Fig. 3). Haplotypes were named according to the subspecies with the most individuals possessing each haplotype. For example, Rog1 is so named for the 35 individuals possessing that haplotype, the majority ($n = 12$), were from the *C. c. rogersi* sample, 8 were from the *C. c. rufa* sample, and 5 were the *C. c. roselaari*, *C. c. piersmai*, and *C. c. islandica* samples. In sharp contrast, the Dunlin network shows very clear sorting into lineages. The Canadian haplotypes are clearly the most divergent, differing from all other haplotypes by at least 14 mutational steps. European haplotypes are differentiated from Siberian, Beringian, and Alaskan haplotypes by at least six mutational steps. And Siberian, Beringian, and Alaskan haplotypes differ by at least three substitutions.

Despite the apparent lack of sorting in the minimum spanning network, Red Knots showed low but significant population differentiation using both conventional *F*-statistics and Exact tests. Four genetically distinct groups were found to correspond to *C. c. canutus*, *C. c. piersmai*, *C. c. rogersi*, and a North American group containing *C. c. roselaari*, *C. c. rufa*, and *C. c. islandica* (Table 2 for F_{ST} summary; Pooled Ex-

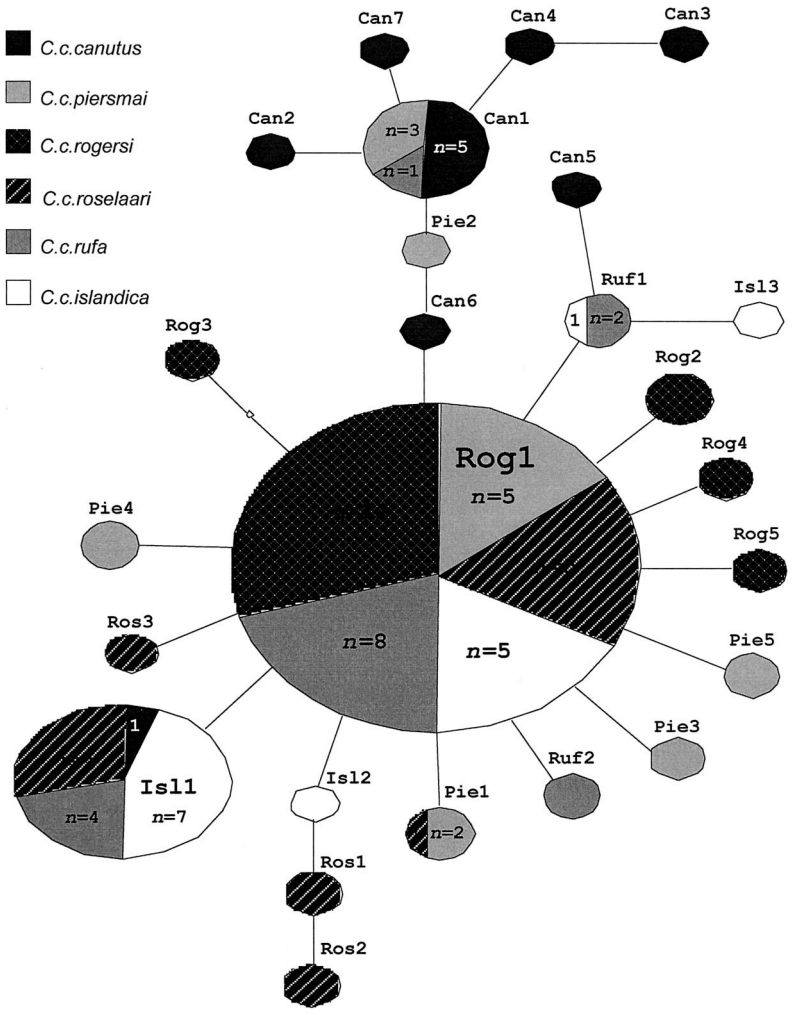

FIGURE 3. Minimum spanning network showing the relationships between haplotypes from mitochondrial control-region sequences of Red Knots. Ovals represent haplotypes and unbroken connecting lines represent a single base pair change between haplotypes. Small open circles on connecting lines represent multiple base-pair changes between haplotypes. Haplotype names are derived from the subspecies predominantly represented: Rog, *C. c. rogersi*, Ruf, *C. c. rufa*; Isl, *C. c. islandica*; Pie, *C. c. piersmai*; and Can, *C. c. canutus*.

act test, $P < 0.001$). Dunlins showed highly significant population differentiation among all lineages (all F_{ST} and Exact tests, $P < 0.001$).

COALESCENT ANALYSES

Posterior probability distributions of θ for both the Red Knot and Dunlin sequences were bell-shaped, indicating a good estimation of this parameter. The θ parameter was used to calculate $N_{ef}\theta = 2N_{ef}\mu$, which was in turn used to calculate the population and gene divergence time estimates ($T = tN_{ef}^{-1}g$).

Female effective population sizes in Red Knot subspecies were quite low and ranged from 4100 in the *C. c. rufa* and *C. c. islandica* comparison to 14 058 in the *C. c. piersmai* and *C. c. canutus* comparison (Table 3). These effective population sizes were on average half those of the major Dunlin lineages which ranged from 10 224 for the Canadian and Alaskan comparison to 44 462 for the European and Beringian comparison (Table 4). Estimates of migration of females ($N_{ef}m$) among subspecies of Red Knots were on average ten times larger than those among lin-

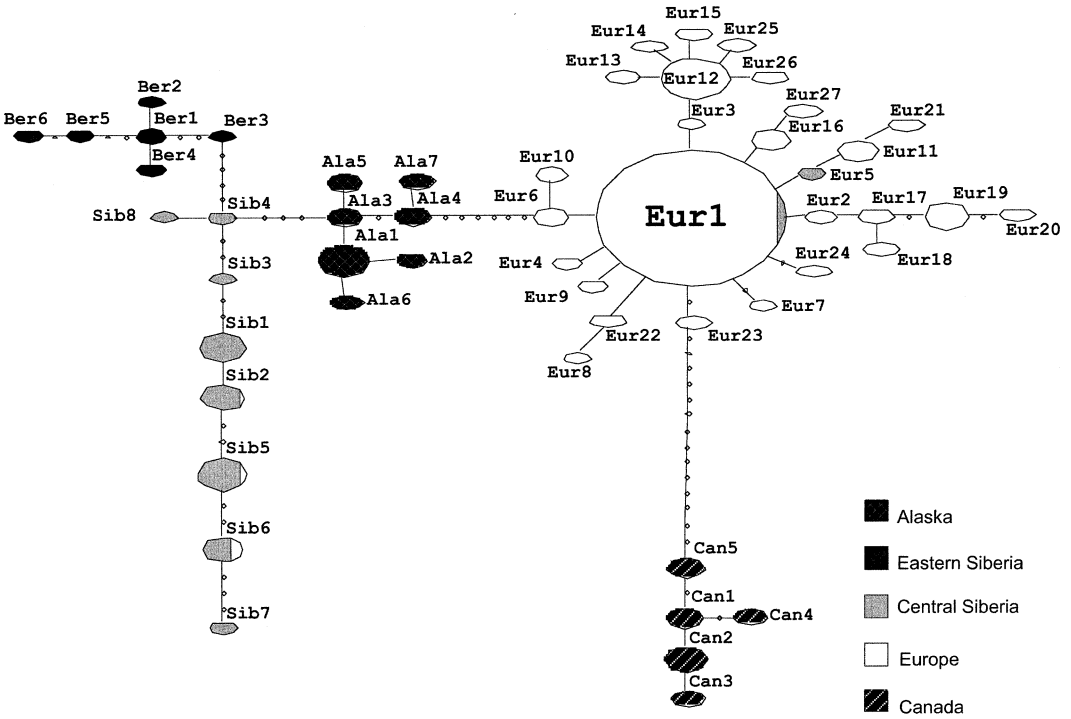

FIGURE 4. Minimum spanning network showing the relationships between haplotypes from mitochondrial control-region sequences of Dunlins. Ovals represent haplotypes and unbroken connecting lines represent a single base pair change between haplotypes. Small open circles on connecting lines represent multiple base pair changes between haplotypes. Haplotype names correspond to those in Wenink et al. (1996) and Wenink and Baker (1996), where Ala, represents samples taken in Alaska; Ber, represents samples taken in eastern Siberia; Sib, represents samples taken in central Siberia; Eur, represents samples taken in Europe; and Can, represents samples taken in Canada.

eages of Dunlins (all Dunlin $N_{ef}m < 0.05$) with Red Knot migration estimates similar to those in European populations of Common Chaffinches (*Fringilla coelebs*, Griswold and Baker 2002) and lower than those in Grasshopper Sparrows (*Ammodramus savannarum*, Bulgin et al. 2003). Estimates of migration were not possible between *C. c. islandica* and *C. c. rufa*, or *C. c. islandica* and *C. c. roselaari* because the posterior probability distributions were ill-conditioned and did not show clear modes. $N_{ef}m$ estimates for Red Knots and Dunlins are shown above the diagonal in Tables 3 and 4, respectively.

Modal estimates of population divergence times in Red Knots indicate that subspecies groups diverged very recently at approximately three time intervals. We calculated these time

TABLE 2. Estimates of F_{ST} for population differentiation (below diagonal) in Red Knots calculated using the Tamura and Nei (1993) model of sequence evolution. Above the diagonal "+" indicates significance at the 0.01 level.

	C.c. canutus	*C.c. islandica*	*C.c. piersmai*	*C.c. rogersi*	*C.c. roselaari*	*C.c. rufa*
C.c. canutus	0	+	+	+	+	+
C.c. islandica	0.19	0	+	+	–	–
C.c. piersmai	0.07	0.12	0	+	–	–
C.c. rogersi	0.27	0.20	0.07	0	+	–
C.c. roselaari	0.17	–0.04	0.08	0.15	0	–
C.c. rufa	0.23	0.005	0.07	0.05	0.002	0

TABLE 3. MDIV estimates of $\theta = 2\ N_{ef}\mu$ and N_{ef} (pairwise estimates of mutation and female effective population size, respectively) below the diagonal, and $M = N_{ef}m$ (migration rates as the number of migrants exchanged between populations per generation), above the diagonal, in Red Knots (*Calidris canutus*). The numbers in parentheses are 95% credibility intervals. Undefined indicates that MDIV analysis produced an ill-conditioned posterior distribution and a modal estimate was not possible.

	C.c. canutus	*C.c. islandica*	*C.c. piersmai*	*C.c. rogersi*	*C.c. roselaari*	*C.c. rufa*
C.c. canutus	–	$M = 0.34$ (0.0–2.6)	$M = 0.69$ (0.0–5.0)	$M = 0.39$ (0.01–2.8)	$M = 0.29$ (0.0–2.5)	$M = 0.32$ (0.0–2.1)
C.c. islandica	$\theta = 2.06$ (0.8–4.9) $N_{ef} = 10\,969$	–	$M = 0.26$ (0.0–2.5)	$M = 0.23$ (0.0–1.5)	undefined	undefined
C.c. piersmai	$\theta = 2.61$ (1.1–6.8) $N_{ef} = 14\,058$	$\theta = 1.65$ (0.7–4.5) $N_{ef} = 8786$	–	$M = 1.39$ (0.1–6.1)	$M = 0.98$ (0.0–10.0)	$M = 0.05$ (0.0–3.0)
C.c. rogersi	$\theta = 2.23$ (1.1–5.7) $N_{ef} = 11\,874$	$\theta = 1.63$ (0.7–4.1) $N_{ef} = 8679$	$\theta = 1.99$ (0.7–4.9) $N_{ef} = 10\,596$	–	$M = 0.61$ (0.0–7.1)	$M = 0.24$ (0.0–6.3)
C.c. roselaari	$\theta = 2.39$ (1.1–58) $N_{ef} = 12\,726$	$\theta = 1.26$ (0.4–3.6) $N_{ef} = 6709$	$\theta = 1.87$ (0.8–5.0) $N_{ef} = 9957$	$\theta = 2.10$ (0.9–5.1) $N_{ef} = 11\,182$	–	$M = 3.68$ (0.0–5.0)
C.c. rufa	$\theta = 1.83$ (0.6–4.2) $N_{ef} = 9744$	$\theta = 0.77$ (0.2–2.5) $N_{ef} = 4100$	$\theta = 1.51$ (0.6–4.7) $N_{ef} = 8040$	$\theta = 1.58$ (0.6–4.5) $N_{ef} = 8413$	$\theta = 1.28$ (0.6–3.6) $N_{ef} = 6816$	–

intervals as the average of population divergence times involved in a particular divergence event. For example, the approximate divergence time of the lineage leading to *C. c. canutus* from the common ancestral population of all other groups is 20 000 (95% CI: 5600–58 000) years ago, an average of the population divergence times for *C. c. canutus* from each of the other subspecies in the first row above the diagonal in Table 5. The other two time intervals represent the divergence of the North American group including *C. c. roselaari*, *C. c. rufa*, and *C. c. islandica* from a Siberian ancestor about 12 000 (95% CI: 3300–40 000) years ago and the split between *C. c. piersmai*, and *C. c. rogersi* about 6500 (95% CI: 1000–23 000) years ago. Modal estimates of divergence times for *C. c. roselaari*, *C. c. rufa*, and *C. c. islandica* were estimated at about 700 years ago; however, 95% credibility intervals included zero (95% CI: 0–5500).

TABLE 4. MDIV estimates of $\theta = 2\ N_{ef}\mu$ and N_{ef} (pairwise estimates of mutation and female effective population size, respectively) below the diagonal, and $M = N_{ef}m$ (migration rates as the number of migrants exchanged between populations per generation), above the diagonal, in Dunlins (*Calidris alpina*). The numbers in parentheses are 95% credibility intervals.

	Alaskan lineage	Beringian lineage	Canadian lineage	European lineage	Siberian lineage
Alaskan lineage	–	$M = 0.04$ (0.0–4.8)	$M = 0.003$ (0.0–0.2)	$M = 0.04$ (0.0–0.4)	$M = 0.03$ (0.0–0.4)
Beringian lineage	$\theta = 2.39$ (1.2–5.6) $N_{ef} = 12\,726$	–	$M = 0.004$ (0.0–0.4)	$M = 0.08$ (0.0–0.6)	$M = 0.04$ (0.0–0.6)
Canadian lineage	$\theta = 1.92$ (1.0–4.3) $N_{ef} = 10\,224$	$\theta = 2.87$ (1.3–6.9) $N_{ef} = 12\,282$	–	$M = 0.03$ (0.0–0.5)	$M = 0.008$ (0.0–0.3)
European lineage	$\theta = 6.32$ (4.1–10.5) $N_{ef} = 33\,653$	$\theta = 8.35$ (1.3–6.9) $N_{ef} = 44\,462$	$\theta = 6.85$ (4.3–12.1) $N_{ef} = 36\,475$	–	$M = 0.03$ (0.0–3.9)
Siberian lineage	$\theta = 2.78$ (1.6–5.3) $N_{ef} = 14\,803$	$\theta = 3.25$ (2.1–7.3) $N_{ef} = 17\,306$	$\theta = 3.24$ (2.2–5.9) $N_{ef} = 17\,252$	$\theta = 6.85$ (4.3–10.6) $N_{ef} = 36\,475$	–

TABLE 5. MDIV estimates of *TMRCA* and gene divergence times (below the diagonal), and *T* and population divergence times (above the diagonal) for pairwise comparisons in Red Knots (*Calidris canutus*). The numbers in parentheses are 95% credibility intervals, with the divergence times, in years before present, listed below. A generation time of 2 years was used to translate divergence times into years before present.

	C.c. canutus	*C.c. islandica*	*C.c. piersmai*
C.c. canutus	–	*T* = 1.04 (0.3–2.7) 22 816	*T* = 0.58 (0.2–2.0) 16 307
C.c. islandica	*TMRCA* = 1.93 42 341	–	*T* = 0.61 (0.2–2.6) 10 719
C.c. piersmai	*TMRCA* = 1.51 42 454	*TMRCA* = 1.95 34 265	–
C.c. rogersi	*TMRCA* = 1.68 39 898	*TMRCA* = 2.01 34 891	*TMRCA* = 1.70 36 028
C.c. roselaari	*TMRCA* = 1.81 46 069	*TMRCA* = 2.17 29 118	*TMRCA* = 1.89 37 639
C.c. rufa	*TMRCA* = 2.05 39 952	*TMRCA* = 2.58 21 157	*TMRCA* = 1.89 30 393

Modal estimates of population divergence times in Dunlins were an order of magnitude greater than those of Red Knots and corresponded well with previously published divergence times calculated from the percentage of sequence divergence after correction for multiple hits (Wenink et al. 1996). The Canadian lineage diverged about 180 000 (95% CI: 38 000–400 000) years ago, the European lineage diverged about 110 000 (95% CI: 53 000–370 000) years ago, and the Siberian, the Beringian, and the Alaskan lineages diverged about 70 000 (95% CI: 21 000–180 000) years ago (Table 6). For both Red Knots and Dunlins, gene divergence times were much greater than population divergence times. This was expected as genes are often older than the populations in which they now occur.

DISCUSSION

DEMOGRAPHIC HISTORY

Red Knots primarily sampled from well-defined staging and wintering areas and Dunlins primarily sampled from breeding areas provide examples of different phylogeographic patterns indicating very different demographic histories. Control region sequences from Red Knots present the classic signature of a species that was recently and severely bottlenecked (Avise 2000). This is evidenced by low genetic diversity in comparison to Dunlins, especially at the species level, a negative Tajima's *D* for the pooled subspecies, a mismatch distribution characteristic of rapid population expansion, and a star-like minimum spanning network. In contrast, control region sequences in Dunlins show the opposite signature of ancient and well-defined mtDNA matrilines corresponding to geographically separated populations, which have not been recently bottlenecked. This is illustrated by high levels of genetic diversity, a nonsignificant Tajima's *D*, a multimodal mismatch-distribution characteristic of two or more well-defined populations, and a deep bifurcating gene tree with well-defined phylogroups which are geographically separated (Wenink et al. 1993).

GENETIC EQUILIBRIUM, GENE FLOW, AND POPULATION STRUCTURE

A comparison between Red Knots and Dunlins is instructive with regards to nonequilibrium conditions (due to shared ancestral polymorphisms) versus current gene flow as the cause for polyphyly and paraphyly in networks. In recently bottlenecked species such as Red Knots, shared ancestral polymorphisms can result in genetic similarity among recently diverged populations, causing misleading estimates of gene flow and a lack of phylogenetic distinctiveness (Bulgin et al. 2003). This problem is exemplified by the subspecies *C. c. roselaari*, *C. c. rufa*, and *C. c. islandica*, which exhibit no detectable lev-

TABLE 5. Extended.

C.c. rogersi	*C.c. roselaari*	*C.c. rufa*
$T = 0.92$	$T = 0.82$	$T = 0.95$
(0.2–2.3)	(0.3–2.7)	(0.2–2.9)
21 849	20 871	18 514
$T = 0.73$	$T = 0.05$	$T = 0.01$
(0.2–2.5)	(0.0–0.5)	(0.0–0.2)
12 672	698	98
$T = 0.31$	$T = 0.57$	$T = 0.86$
(0.05–1.1)	(0.1–1.7)	(0.2–2.3)
6570	11 351	13 830
–	$T = 0.46$	$T = 0.64$
	(0.2–2.2)	(0.2–2.0)
	10 288	10 769
$TMRCA = 1.83$	–	$T = 0.09$
40 927		(0.0–0.6)
		1254
$TMRCA = 1.92$	$TMRCA = 2.33$	–
32 307	31 761	

els of genetic differentiation even in the fast-evolving control region. Furthermore, pairwise estimates of N_{ef} m in these subspecies could not be estimated with the coalescent model as the posterior probability surfaces were so ill-conditioned that no modes were present. This, along with negative F_{ST} values, could be interpreted as either a product of shared ancestral polymorphisms, or unrealistically as infinite amounts of gene flow.

The time (in generations) needed for F_{ST} to reach half way from an old to a new equilibrium is $t(0.5) = \ln(1\ 2^{-1})\ \{\ln[(1 - m)^2(1 - 1\ 2N_e^{-1})]\}^{-1}$ (Whitlock 1992). Substituting our estimates of N_{ef} and m for our most divergent groups of Red Knots, *C. c. canutus* and *C. c. islandica*, produced a time of 12 886 years to reach half way to equilibrium. Because the relationship between F_{ST} and time to genetic equilibrium is nonlinear, the total time to equilibrium is more than twice the time calculated above. Using the curve in Figure 3 from Whitlock and McCauley (1999), we can extrapolate that the time for F_{ST} to reach 95% equilibrium for these subspecies is about 130 000 years. This is much longer than any of our estimated population divergence times including their broad 95% confidence intervals. Clearly Knot subspecies are not in equilibrium, and their lack of genetic distinctiveness and the polyphyly in their gene tree is most likely due to shared ancestral polymorphisms and not high levels of gene flow.

Contrary to Red Knots, Dunlins exemplify a species that has probably reached genetic equilibrium. The substitution of our estimates of N_{ef} and m for the Canadian and Siberian lineages in Dunlins into Whitlock's (1992) equation produces a time halfway to equilibrium of 46 350 years. The time to 95% equilibrium can be extrapolated to about 200 000 years (Whitlock and McCauley 1999), which is close to our estimated population divergence time for these two lineages. These calculations indicate that Dunlins are most likely in genetic equilibrium as enough time has passed for monophyly to evolve among lineages in this species. Because Dunlin lineages are in genetic equilibrium, the small amount of mixing found in their haplotype network should be interpreted as a small number of contemporary migrants, and not as shared ancestral polymorphism. The mixing between the European and Siberian phylogroups is probably caused by occasional migrants and interbreeding in the geographically restricted zones of overlap between these groups (Wenink et al. 1996, Wennerberg et al. 1999, Wennerberg 2001).

While the six currently recognized subspecies of Red Knots cannot be distinguished by their control region sequences, they are beginning to sort into different lineages and it would be in-

TABLE 6. MDIV estimates of *TMRCA* and gene divergence times (below the diagonal), and *T* and population divergence times (above the diagonal) for pairwise comparisons in Dunlins (*Calidris alpina*). The numbers in parentheses are 95% credibility intervals and the numbers below the credibility intervals are divergence times in years before present. A generation time of 2 years was used to translate divergence times into years before present.

	Alaskan lineage	Beringian lineage
Alaskan lineage	–	*T* = 3.08 (0.8–8.5) 78 394
Beringian lineage	*TMRCA* = 3.83 97 483	–
Canadian lineage	*TMRCA* = 11.1 226 965	*TMRCA* = 7.74 236 569
European lineage	*TMRCA* = 2.27 152 784	*TMRCA* = 1.85 164 510
Siberian lineage	*TMRCA* = 2.77 82 009	*TMRCA* = 2.70 93 450

advisable to lump them into a single evolutionary unit on genetic grounds alone. Given the passage of time, evolution of neutral genetic markers in Red Knots should catch up with morphological and behavioral characters such as size and plumage differences, different migration routes, separate breeding grounds, and different molt schedules to more clearly distinguish subspecies (Davidson and Wilson 1992, Piersma and Davidson 1992, Morrison and Harrington 1992, Tomkovich 1992, Nebel et al. 2000, Tomkovich 2001).

RECONSTRUCTING HISTORICAL BIOGEOGRAPHY

Coalescent analyses in Red Knots suggest four emergent lineages which diverged about 20 000, 12 000 and 6500 years ago, suggesting that divergence of extant subspecies probably originated near or after the glacial maximum of the Wisconsinan or Weichselian glaciation, 22 000 to 18 000 years ago. These dates are suggestive of an eastwards expansion of Red Knots from a single population that survived the last glacial maximum breeding in the unglaciated regions of central and eastern Eurasia. The very recent population divergence between lineages breeding in North America and older divergence dates from Eurasian breeding lineages suggest that breeding grounds in the high Canadian Arctic and Greenland were established by the eastward expansion of a North American ancestral stock rather than recent colonization from Europe.

The extreme cold and arid conditions of the last glacial maximum may have caused the first population divergence approximately 20 000 years ago (Adams 1997). Later divergences may have been caused by expansion via Beringia into North America as the Laurentide and Cordilleran ice sheets began to melt about 12 000 years ago (Pielou 1991, Adams 1997), and by geographic isolation of breeding populations during the climatic optimum of the Holocene between 9000 and 6000 years ago when the area of available breeding tundra for knots was drastically reduced (Adams 1997).

The hypothesis of sequential eastward expansion of Red Knots is consistent with the loss of nucleotide diversity in daughter populations. In Red Knots, the *C. c. canutus* group shows the highest level of nucleotide diversity, consistent with a possible basal divergence. *C. c. roselaari* and *C. c. piersmai* have intermediate levels of genetic diversity as expected by their intermediate divergence times. Finally, *C. c. rogersi*, *C. c. islandica*, and *C. c. rufa* have the lowest levels of diversity indicating that they diverged most recently, and have not had sufficient time to recover genetic variation lost through serial founder effects.

Dunlins have a demographic history that is very different from that of Red Knots with coalescent times dating back about 180 000 years, indicating that Dunlins were last bottlenecked to a single ancestral population during the Illinoian or Saale glaciation 215 000 to 135 000 years ago

TABLE 6. Extended.

Canadian lineage	European lineage	Siberian lineage
$T = 8.76$ (1.2–19.0) 179 118	$T = 1.62$ (0.8–4.5) 109 035	$T = 1.97$ (0.8–5.5) 58 324
$T = 6.36$ (1.0–14.0) 194 390	$T = 1.29$ (0.6–4.0) 114 712	$T = 1.95$ (0.6–5.0) 67 492
–	$T = 2.34$ (0.8–5.0) 170 703	$T = 5.68$ (1.1–13.0) 195 987
$TMRCA = 3.11$ 226 874	–	$T = 1.53$ (0.7–4.1) 111 613
$TMRCA = 7.19$ 248 089	$TMRCA = 2.25$ 164 137	–

(Dansgaard et al. 1993). Our date of population divergence is slightly younger than that given by Wenink et al. (1996) owing to the fact that we are using dates for population divergence and not gene divergence. During this time, a tundra refugium existed south of the North American ice sheet (Morgan et al. 1983, Morgan 1987) and may have served as a refuge for a completely isolated Canadian breeding population.

We caution that our reconstructions of biogeography are hypotheses based on population divergence times with broad 95% confidence intervals, and they could be affected by errors in estimating mutation rates and intraspecific rate variation in the molecular clock. Nevertheless, the divergence times correlate well with the approximate timing of major events during the late Pleistocene. These hypotheses may be further tested by the examination of biogeography in other species with demographic histories similar to Red Knots or Dunlins. For example, the biogeography of Ruddy Turnstones (*Arenaria interpres*) and Sanderlings (*Calidris alba*) would be particularly instructive for testing the eastwards expansion of Red Knots and the engineering of the *C. c. islandica* flyway. Both of these species are high arctic breeders with circumpolar breeding ranges, and Ruddy Turnstones show low genetic diversity suggestive of a recent population bottleneck like that seen in Red Knots (Baker et al. 1994). Very recent population divergence between lineages breeding in North America and older divergence dates from Eurasian breeding lineages would suggest that breeding grounds in the high Canadian Arctic and Greenland became established from the expansion of a North American ancestral breeding population that later pioneered a migratory route to northwest Europe, rather than a northwest expansion of a European wintering ancestor. This cross-species examination would provide either support for our hypotheses through biogeographic concordance, or suggest idiosyncratic population histories unrelated to major climatic shifts in the past.

Another important test would be in assaying sequence divergence in other unlinked genes for both Red Knots and Dunlins. This has the additional benefit of reducing error in the estimates of coalescent times (Edwards and Beerli 2000). We present data for the mitochondrial control region which is only a small portion of the genome, and our estimates are restricted to matrilines. Unfortunately, a multigene test may prove to be difficult as genes with comparably fast rates of evolution will be needed to resolve intraspecific groupings, especially in Red Knots.

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APPENDIX A. Sample locations, dates, and sample sizes for Red Knots (*Calidris canutus*) used in this study.

Location collected	Type of site	Date collected	Subspecies	*n*
South Carolina, USA	Wintering	16 Apr. 2000	*C.c. roselaari*	6
Georgia, USA	Wintering	17 Oct. 1999	*C.c. roselaari*	9
Tierra del Fuego, Argentina	Wintering	4 Dec. 2000	*C.c. rufa*	8
Southampton Island, Canada	Breeding	5–7 Jul. 2000	*C.c. rufa*	7
Broome, Western Australia, Australia	Wintering	24 Mar. 1996	*C.c. piersmai*	15
Ellesmere Island, Canada	Breeding	1 Jun. 1990	*C.c. islandica*	9
Dutch Wadden Sea, Netherlands	Staging	27 Oct. 1995	*C.c. islandica*	1
Dutch Wadden Sea, Netherlands	Staging	8–9 Feb. 1997	*C.c. islandica*	5
Taymyr Peninsula, Russia	Breeding	6–10 Jul. 1994	*C.c. canutus*	6
Cape Province, South Africa	Wintering	30 Nov. 1991	*C.c. canutus*	6
Barry Beach, Victoria, Australia	Wintering	2 Apr. 2002	*C.c. rogersi*	18
Corner Inlet, Victoria, Australia	Wintering	25 Jul. 2002	*C.c. rogersi*	1

APPENDIX B. Variable sites in 25 Red Knot (*Calidris canutus*) haplotypes. Variation is represented with respect to the first haplotype listed and identities are indicated by dots. Sites are numbered according to the complete control region sequence presented in Buehler and Baker (2003). Haplotype names are derived from the subspecies predominantly represented: Rog, *C.c. rogersi*; Ruf, *C.c. rufa*; Isl, *C.c. islandica*; Pie, *C.c. piersmai*; and Can, *C.c. canutus*.

Haplotype	*n*	112233333344 395813467902 887528969530	111 778889011 780299902 827799844
1 Rog1	35	TTGTCTGTCACA	CATATTAGG
2 Rog2	3	..A.....	.....
3 Rog3	1	.C.....	......G..
4 Rog4	1	.....	.....A
5 Rog5	1	.......C....	.....
6 Ruf1	3	......A.....	.....
7 Ruf2	1	.....T...	.....
8 Is11	18	.....	.....C...
9 Is12	1	.....	.G.....
10 Is13	1	......A.....	....C....
11 Ros1	1	A.....	.G.....
12 Ros2	1	A.....	.GC.....
13 Ros3	1	.....	T.....
14 Pie1	3	.....C.....	.....
15 Pie2	1	.....T.	...G.....
16 Pie3	1	...C.....	.....
17 Pie4	2	.....	.....A.
18 Pie5	1	.....G..	.....
19 Can1	9	.....	...G.....
20 Can2	1	....T.....	...G.....
21 Can3	1	......A....G	...G.....
22 Can4	1	.....G	...G.....
23 Can5	1	......A.....	...G.....
24 Can6	1	.....T.	.....
25 Can7	1	.C.....	...G.....

APPENDIX C. Variable sites among 53 Dunlin (*Calidris alpina*) haplotypes. Variation is represented with respect to the first haplotype listed and identities are indicated by dots. Sites are numbered according to the Dunlin control region sequence (Wenink et al. 1993). Haplotype names correspond to those in Wenink et al. (1996) and Wenink and Baker (1996).

Haplotype	*n*	11111112222222222222233333333333 5566677777737777 25779990122222555577822222334566 75706611122334444 30383681701347578956934567494812 75427802907084 89
1 ALA1	12	AACATCACAGTACACCTGCTATTAACCTTCGAG GCCT-AATCCAGTGT
2 ALA2	2	...G..... .....
3 ALA3	10	.....A..... .....
4 ALA4	4	..T.....A..... .....G.....
5 ALA5	3	.....A.....G..... .....
6 ALA6	1	..T..... .....
7 ALA7	1	G.T.....A..... .....G.....
8 BER1	2	......G..A....T..A..G.....T....GA .....G.....
9 BER2	1	......G..A....T..A..G.....T....GA .....G...T.....
10 BER3	1	......G..A.......A..G.....GA .....G..T.....
11 BER4	1	....C.G..A....T..A..G.....T....GA .....G.....
12 BER5	1	G.....A....T..A..G.....GA .....G.....
13 BER6	1	G.....G..A....T..A..G.....A .....G.....
14 SIB1	10	......G..A.....C..C..T..... .....G..T.....
15 SIB2	9	......G.....C..C..T..... .....G..T.....
16 SIB3	1	......G..A.....C..... .T...G..T.....
17 SIB4	1	......G..A.....C.... .....G..T.....
18 SIB5	12	......G....G.....C.....TT..... .....G..T.....
19 SIB6	7	.G....G..A.G.....C.....TT..... .....G..T.....C
20 SIB7	1	.G....G..A.G.....G. .....G..T.....C
21 SIB8	1	......G..A.....T.C.... .....G..T.....
22 EUR1	57	..T.....A.....AC...G.....CTA.A .....G..T.....
23 EUR2	2	..T...G..A.....AC...G.....CTA.A .....G..T.....
24 EUR3	1	..T....T.A.....AC...G.....CTA.A .....G..T.....
25 EUR4	1	..T.....A...G.AC...G.....CTA.A .....G..T.....
26 EUR5	1	..T.....A.....AC...G....T..CTA.A .....G..T.....
27 EUR6	3	..T.....A.....AC...G.....CTA.. .....G..T.....
28 EUR7	1	..T.....AC..CG.....CTA.A .....G..T.....
29 EUR8	1	..T.....AC...G...G...CTA.A .....G..T.....
30 EUR9	1	.....A.....AC...G.....CTA.A .....G..TT.....
31 EUR10	2	..T.....A...G.AC...G.....CTA.. .....G..T.....
32 EUR11	4	..T.....A.....AC.....T..CTA.A .....G..T.....
33 EUR12	11	..T..T.T.A.....AC...G.....CTA.A .....G..T.....
34 EUR13	1	..T..T.T.A.....AC...G.....T.CTA.A .....G..T.....
35 EUR14	1	..T..T.T.A.....AC...G.....CTA.. .....G..T.....
36 EUR15	1	..T..T.T.A....TAC..CG.....CTA.A .....G..T.....
37 EUR16	5	..T.....A.....AC...G.....CTA.A .....G-CT.....
38 EUR17	2	..T...G..A.....AC...G.....CTA.A .....G-CT.....
39 EUR18	1	......G..A.....AC...G.....CTA.A .....G-CT.....
40 EUR19	6	..T...G.....AC...G.....CTA.A .....G-CT...G..
41 EUR20	1	..T.....AC...G.....CTA.. .....G-CT...G..
42 EUR21	1	..T.....A.....AC.....CTA.A .....G..T.....
43 EUR22	1	..T.....A.....AC...G...G...CTA.A .....G..T.....
44 EUR23	1	.....A.....AC...G.....T.CTA.A .....G..T.....
45 EUR24	1	..T.....A.....AC...G.....CTA.A .....G..T....AG
46 EUR25	1	..T..TGT.A.....AC...G.....CTA.A .....G..T.....
47 EUR26	1	..T..T.T.A.....AC...G..G....CTA.A .....G..T.....
48 EUR27	1	..T.....A.....AC...G.....CTA.A ..A..G-CT.....
49 CAN1	4	.....TACCT..GCAAC.C....TAC.A.A A..CGG..T.GA...
50 CAN2	6	.....TACCT..GCAAC.C....TAC.A.A A..CGG..T..A...
51 CAN3	1	.....CACCT..GCAAC.C....TAC.A.A A..CGG..T..A...
52 CAN4	1	.....CACCT..ACAAC.C....TAC.A.A A..CGG..T.GA...
53 CAN5	4	.....TACCT..ACAAC.C....TACTA.A A..CGG..T.GA...